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Genetic variation of *Ginkgo biloba* L. (Ginkgoaceae) based on cpDNA PCR-RFLPs: inference of glacial refugia

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Ginkgo biloba, a famous living fossil, is the sole survivor of the genus *Ginkgo*. To make inferences about the glacial refugia that harbored *G. biloba*, we examined the genetic structure of eight potential refugial populations and plantations using chloroplast DNA (cpDNA) with eight size variants in the *trn*K1-*trn*K2 fragment. The data consist of haplotypes from 158 trees collected from eight localities. The majority of the cpDNA haplotypes are restricted to minor portions of the geographical range. Our results suggest that refugia of *G. biloba* were located in southwestern China. This area is a current biodiversity hotspot of global importance, and may have been protected from the extremes of climatic fluctuations during the Pleistocene. The *Ginkgos* on West Tianmu Mountain, which were previously considered to be wild by many researchers, may, instead, have been introduced by Buddhist monks.
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Introduction

The distribution and genetic diversity of plant species have been deeply modified by Pleistocene glaciations (Comes and Kadereit, 1998; Hewitt, 1999, 2004; Newton *et al*, 1999; Abbott and Brochmann, 2003). During glaciations, the ranges of species changed and some ancestral populations became extinct, and recolonization subsequently occurred during interglacial periods (Widmer and Lexer, 2001). The species genomes were dramatically influenced by these processes, and molecular markers can be used to infer the phylogeographic pattern of species in response to the Pleistocene climatic changes (Newton *et al*, 1999; Shen *et al*, 2002; Abbott and Comes, 2004).

In contrast to intense investigations on plants of Europe and northern America (Hewitt, 2000; Shen *et al*, 2002), understanding of the effects of past climatic events on population structure and phylogeny of species in Asia is relatively limited (Szmidt and Wang, 1993; Wang and Szmidt, 1994; Szmidt *et al*, 1996; Chen *et al*, 1997; Tomaru *et al*, 1997; Lin, 2001; Huang *et al*, 2002; Lu *et al*, 2002; Okaura and Harada, 2002; Aoki *et al*, 2004). The Eurasian ice sheets covered millions of square kilometers of the continent in the late Pleistocene period (Svendsen *et al*, 1999). It is accepted that, during the Quaternary, mountains over 3500 m in western China became glaciated, but there is disagreement over whether the lower central and eastern mountains (<3000 m) were glaciated (Shi *et al*, 1989). Temperatures are estimated to have been 5–13°C lower than at present in China (Shi *et al*, 1989): low enough to have profound impact on plants (Wang and Liu, 1994).

Ginkgos (Ginkgoaceae) have a history dating back approximately to the early Permian (~280 MA) (Willis and McElwain, 2002). At the height of their global radiation, fossil evidence indicates that there were at least 16 genera and that they formed a significant part of the world vegetation (Willis and McElwain, 2002). Today, they are represented by a single species, *Ginkgo biloba*, which is endemic to China. This species was listed as a rare species in the 1997 IUCN red list of threatened plants (http://www.unep-wcmc.org/) and listed in the red list of endangered plant species of China (Fu and Jin, 1992). Thus, it should receive effective conservation. However, the existence of natural *G. biloba* populations is disputed and the refugia during Pleistocene glaciations are not known (Liang and Li, 2001). The West Tianmu Mountain of Zhejiang Province is one likely refugium (Liang and Li, 2001; Lin and Zhang, 2004). Recently, several papers have suggested that some other sites had native populations, based on plant community surveys and human settlement history (Zhou *et al*, 1982; Xiang and Xiang, 1997; Xiang *et al*, 1998; Li *et al*, 1999), although this evidence might be considered inconclusive.

Molecular markers have proved to be useful tools for identifying refugia and tracking postglacial migration routes of plant species. Chloroplast DNA (cpDNA) is maternally inherited in most flowering plants and some gymnosperms (such as *Ginkgo*, *Cycas* and *Ephedra*) (Mogensen, 1996). It therefore provides a seed-specific marker. Maternally inherited markers tend to display

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highly structured geographical distribution because of the lower potential for dispersal by seeds than by pollen (Newton *et al*, 1999). It is consequently easier to elucidate the refugia and migration history of plant species using maternally inherited markers (rather than biparently inherited ones) (Newton *et al*, 1999). In the present paper, we try to understand the cpDNA haplotype diversity of *G. biloba*, and to infer the potential refugia.

Materials and methods

Species and sampling

G. biloba is a long-lived deciduous species. It occurs through most of mainland China and many other countries. However, most trees have been planted for ornamental or medical purposes. Several sites were reported to have natural *Ginkgo* based on plant community surveys or/and the history of human settlement (Table 1). We will call them 'natural' populations because no convincing evidence exists to the contrary. We sampled populations covering these sites (six populations) as well as two additional planted populations (Table 1, Figure 1). They can be divided into three regions: southwestern China (JF, TL and SP), eastern China (TM, CX and YS) and central China (LY and XA).

Buds were collected from trees in West Tianmu Mountain (TM) and brought fresh to the laboratory for DNA extraction, while leaves were collected from individuals of the other seven populations and dried using silica gel has described previously (Chase and Hills, 1991). In all, 11–27 trees were sampled from each location. In order to avoid sampling recently planted trees, we sampled those with a DBH (diameter at breast-height) as large as possible. The DBH of the sampled trees ranged from 39 to 210 cm, of which only 10 (three in SP and TM and four in XA population) were less than 50 cm (Table 1).

DNA extraction

Total DNA was extracted using a miniprep CTAB protocol modified from Doyle and Doyle (1987). Dry leaves were ground to powder with a cold grinder, suspended in 750 µl 60°C CTAB 2 × buffer and incubated for 90 min. After chloroform isoamyl extraction, the aqueous phase was collected and digested with RNase A for 30 min. Finally, the nucleic acid was precipitated with 100% ethanol and resuspended in TE buffer.

PCR-RFLP of cpDNA

PCR assays were performed in 50 µl total volume containing 0.2 µM of each primer, 10 mM KCl, 8 mM $(NH_4)_2SO_4$, 10 mM Tris-HCl (pH 9.0), 2.5 mM $MgCl_2$, 200 µM dNTPs, 2 U of *Taq* DNA polymerase and 40 ng template DNA. In total, 16 pairs of universal primers for noncoding regions of cpDNA (Taberlet *et al*, 1991; Demesure *et al*, 1995; Dumolin-Lapegue *et al*, 1997b) were used in initial screening. No product was obtained using primers for *trnf*M-*psa*A, *trn*V-*rbc*L and *trn*F-*trn*V (Dumolin-Lapegue *et al*, 1997b).

Fragments of *trn*H-*trn*K, *trn*C-*trn*D, *psa*A-*trn*S, *trn*S-*trn*T, *trn*K-*trn*Q, *trn*Q-*trn*R, *rpo*C-*trn*C, *trn*T-*psb*C and *trn*T-*trn*F were of low quality and not suitable for RFLP analysis. The other four pairs of primers for *trn*K1-*trn*K2, *trn*D-*trn*T, *psb*C-*trn*S and *trn*S-*trnf*M produced fragments of suitable quality, which then were digested with the endonucleases *Rsa*I, *Hinf*I and *Hae*III, respectively. After digestion at 37°C for 6 h, the products were resolved electrophoretically on either 2% agarose gels or 8% polyacrylamide gels stained with ethidium bromide and photographed using Bio-Rad Gel Doc^{2000}.

Data analysis

Diversity measures were calculated directly on the haplotype frequencies using Lewis and Zaykin's (2000) GDA program (version 1.0 d15). The number of

Table 1 Location and sample size of *G. biloba* populations in the present study

Population	*Abbreviation*	*Sample size*	*DBH (cm)*	*Longitude*	*Latitude*	*Altitude (m)*	*Description*
Luoyang of Suizhou City, Hubei Province	LY	16	60–210	113°19′E	31°26′N	134–198	Native community (Zhou *et al*, 1982)
Xiaan of Xixia County, He'nan Province	XA	20	39–156	111°47′E	33°32′N	776–845	Natural population reported by the media
Jinfou Mountain of Nanchuan County, Chongqing Municipality	JF	24	58–151[a]	107°11′E	29°03′N	890–1301	Natural population (Li *et al*, 1999)
Shaping of Guiyang City, Guizhou Province	SP	20	40–198	106°48′E	26°15′N	1272	Natural population (Xiang and Xiang, 1999)
Tuole of Pan County, Guizhou Province	TL	24	56–203	104°32′E	25°36′N	1632	Natural population (Xiang *et al*, 2003)
Badujie of Changxing County, Zhejiang Province	CX	16	60–94	119°42′E	31°01′N	127–210	Plantations
Yushan of Yongxiu County, Jiangxi Province	YS	11	ND	ND	ND	ND	Cultivated individuals in a temple
West Tianmu Mountain of Lin'an City, Zhejiang Province	TM	27	46–135	119°27′E	30°19′N	440–1193	Natural population (Hu, 1954; Lin and Zhang, 2004)

[a]An individual's DBH was 3.69 m in 1950s. The trunk was destroyed by a fire in 1960s and sprouted again after the fire. We sampled leaves of sprouts of the tree.
ND: not determined.

haplotypes and haplotype diversity ($\hat{h}$) were calculated for each population and at region and species level, according to Nei (1987). Gene flow among populations was characterized as Nm, the estimated number of female migrants per generation between populations, using the formula $F_{ST} = 1/(1+2Nm)$ where N is the female effective population size and m is the female migration rate.

Results

With the enzymes *Rsa*I, *Hinf*I and *Hae*III, no variation was observed in restriction patterns of the amplification products of primers for *trn*D-*trn*T, *psb*C-*trn*S and *trn*S-*trn*fM. Variants were observed for *trn*K1-*trn*K2 products when digested by *Rsa*I, *Hinf*I and *Hae*III (Table 2). All the variants are caused by point mutations. Eight haplotypes were defined (*A*–*H*). Three were common, with frequencies of 0.474 (haplotype E), 0.314 (haplotype B) and 0.160 (haplotype A), four were rare (frequencies equal to 0.006 for haplotype C, D, G and H), and haplotype F was infrequent, with a frequency of 0.026.

Population JF has the highest effective number of haplotypes (2.53), followed by CX (2.51) and TL (1.52). Similar trends are observed for the actual numbers of haplotypes (Table 3). Haplotype diversity ($\hat{h}$) is greatest in population CX, followed by JF and TL (Table 3). One other population (LY), almost fixed for haplotype E, has one additional variant ($\hat{h} = 0.121$), and the remaining four population samples are fixed for one haplotype. The mean $\hat{h}$ is 0.214 and $\hat{h}$ at the species level is 0.652. At the regional level, there are 7, 4 and 3 haplotypes for southwestern, eastern and central China, respectively. The value of $\hat{h}$ ranges from 0.246 (eastern China) to 0.685 (southwestern China) with a mean of 0.485. The overall value of θ is 0.676 (Walter and Epperson, 2001), and the estimate of Nm is 0.240.

Nei's (1978) genetic distance reflects the same pattern of haplotype differentiation between pairs of populations (Table 4).

Discussion

cpDNA polymorphism in *G. biloba*

With three different restriction enzymes, eight different cpDNA haplotypes were found in 158 individuals (Table 2). The level of genetic variation is moderate

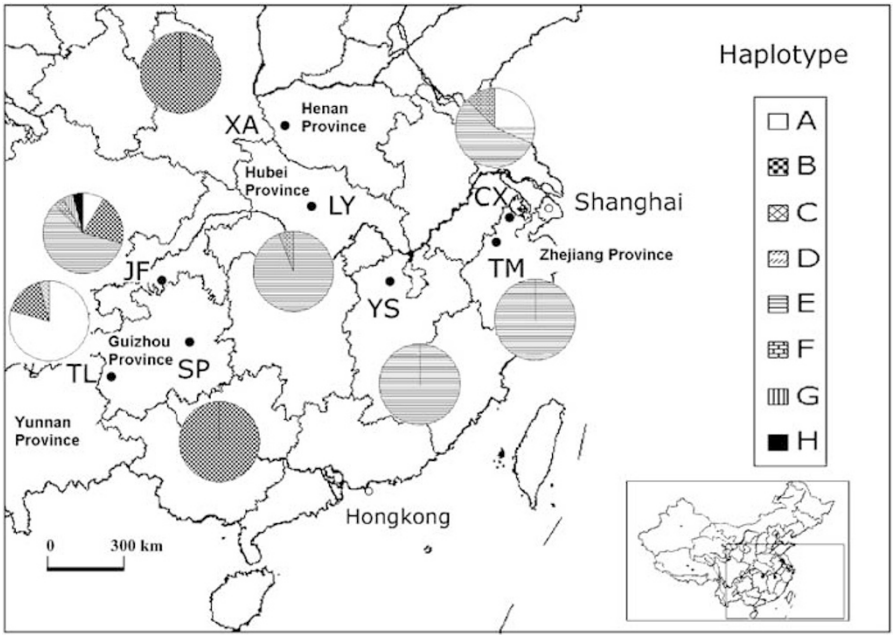

Figure 1 Geographic locations of eight populations of *G. biloba* in China. TL: Tuole of Pan County, Guizhou Province; CX: Changxing County, Zhejiang Province; LY: Luoyang of Suizhou City, Hubei Province; YS: Yushan of Yongxiu County, Jiangxi Province; SP: Shaping of Guiyang City, Guizhou Province; TM: West Tianmu Mountain of Linan City, Zhejiang Province; XA: Xiaan of Xixia County, He'nan Province; JF: Jinfou Mountain of Nanchuan County, Chongqing Municipality.

Table 2 Haplotype definitions, with the restriction profiles of *trn*K1-*trn*K2 by each endonuclease, and measures of differentiation (θ) in the study populations of *G. biloba*

Endonuclease	*Haplotype*								
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*Combined*
*Rsa*I	1[a]	2	1	1	1	1	2	2	
*Hinf*I	1	2	1	2	1	2	1	1	
*Hae*III	3	2	2	2	1	1	2	1	
θ	0.5943	0.7956	−0.0116	0.0135	0.7586	0.0232	−0.0116	−0.0116	0.6764

[a]1, 2 and 3 is code of the restriction profile.

Table 3 Frequencies and diversity of haplotypes within study populations of *G. biloba*

cpDNA types	*Population*								*Total/mean*	*Total/mean without YS and CX*
	JF[a]	*TL*	*SP*	*TM*	*YS*	*CX*	*LY*	*XA*		
A	2	19	0	0	0	4	0	0	25	21
B	5	4	20	0	0	0	0	20	49	49
C	0	1	0	0	0	0	0	0	1	1
D	0	0	0	0	0	1	0	0	1	0
E	14	0	0	25	11	9	15	0	74	54
F	1	0	0	0	0	2	1	0	4	2
G	1	0	0	0	0	0	0	0	1	1
H	1	0	0	0	0	0	0	0	1	1
No. of plants	24	24	20	25	11	16	16	20	156	115
A[b]	6	3	1	1	1	4	2	1	8/2.375	7/2.333
A_E[c]	2.53	1.52	1	1	1	2.51	1.13	1	2.86/1.46	2.97/1.36
$\hat{h}$[d]	0.617 (0.067)	0.351 (0.077)	0	0	0	0.621 (0.070)	0.121 (0.075)	0	0.652/0.214	0.566/0.182

[a]Population codes are the same as in Table 1.
[b]Number of different haplotypes.
[c]Effective number of haplotypes.
[d]Haplotype diversity. Numerals in brackets are standard errors.

Table 4 Genetic distance[a] based on haplotype frequencies

	JF[b]	*TL*	*SP*	*TM*	*YS*	*CX*	*LY*	*XA*
JF	—	1.5995	1.0887	0.0591	0.0591	0.0730	0.0544	1.0887
TL		—	1.5755	∞	∞	0.9186	∞	1.5755
SP			—	∞	∞	∞	∞	0.000
TM				—	0.0000	0.0903	0.0001	∞
YS					—	0.0903	0.0001	∞
CX						—	0.0756	∞
LY							—	∞
XA								—

[a]Pairs denoted by ∞ were fixed for different haplotypes, and thus Nei's (1978) measure is undefined. Values of 0 occurred when pairs were fixed for identical haplotypes.
[b]Codes of population are the same as in Table 1.

compared to that of other plants, for example, three haplotypes in 52 *Silene hifacensis* individuals (Prentice *et al*, 2003), 10 cpDNA haplotypes in 188 *Beta vulgaris* ssp. *maritima* individuals (Forcioli *et al*, 1998), 13 haplotypes in 217 *Alnus glutinosa* individuals (King and Ferris, 1998) and 23 haplotypes in 1412 white oaks (Dumolin-Lapegue *et al*, 1997a).

At the regional level, southeastern China has the highest haplotype diversity and central China the lowest. Genetic variation at the population level is much more variable. Population JF has as many as six haplotypes, while the other four populations have only one haplotype. It is intriguing that CX has more haplotypes than some other sites and has the highest haplotype diversity (0.621). The CX population is believed to have been transplanted from other sites, hence artificial admixture can explain the high diversity, if introduced individuals were drawn from several populations (Wade and McCauley, 1988). High genetic diversity in nonrefugia population has been observed in other plant species. *Pinus resinosa*, for example, shows the highest diversity in previously glaciated areas (Walter and Epperson, 2001) due to the natural postglacial recolonization from different refugia.

It has been shown theoretically and empirically that the level of genetic differentiation among populations is expected to be higher for maternally inherited cpDNA markers than for biparentally inherited nuclear genes (Ennos, 1994; Raspe *et al*, 2000). In accordance with this expectation, a G_{ST} of 0.161 was observed for RAPD markers in *G. biloba* (Fan *et al*, 2004), whereas a much higher level of differentiation ($\theta = 0.676$) was found for cpDNA haplotypes in the present study. Limited gene flow and drift may contribute to the difference (Raspe *et al*, 2000).

Glacial refugia of *G. biloba*

The earliest known fossil of *Ginkgo* is from the Middle Jurassic epoch of 170 Myr ago and evolutionary history became clear with a recent find (Zhou and Zheng, 2003). East Asia provided the later habitats of *Ginkgo* species, and fossils have been found from the Oligocene, Miocene, Pliocene and early Pleistocene in Japan (Uemura, 1997). It has been suggested that West Tianmu Mountain was the refuge of *G. biloba* during the last glaciations, although there has been some disagreement (Liang and Li, 2001; Lin and Zhang, 2004) due to lack of a detailed fossil record (Zhou, 2003).

The cpDNA variation reported here indicates strong genetic differentiation and shows that the spread of *G. biloba* since the last glaciation does not coincide with

previous expectations. The pattern suggests that southwestern China was a refugium for *G. biloba*. Seven of the eight haplotypes were found there and three of them were endemic to this region.

It would not be too surprising if the refugium of *G. biloba* was located in SW China during the Pleistocene ice ages. SW China is located southeast of the Tibetan Plateau and south to the Qing Mountains. The plateau and mountains form a natural division between the temperate zone and subtropical zone in mainland China. Consequently, SW China was less affected by cold air from Siberia during the glaciations. This region has the greatest biodiversity in China due to the relatively stable environment and diverse topography. It is also one of the 25 global biodiversity hotspots (Myers *et al*, 2000). The Yunnan Province, for example, has been estimated to harbor more than 15 000 vascular plant species. Many other living fossils are also found there. For example, *Cathaya argyrophylla*, *Tetracentron sinense* and natural *Metasequoia glyptostroboides* populations have been found in remote mountains of this region. Wang and Liu (1994) also suggested that this region is an important refugium for species surviving the Pleistocene glaciations.

The most likely refugium is sited in Jinfoshan, at the boundary of Chonqing Municiplity and Guizhou Province, as suggested by the current occurrence of ancient *Ginkgo* trees. Recently, a natural-like *Ginkgo* forest was reported in Jinfoshan by Li *et al* (1999) where there are 70 trees with DBH larger than 1 m and eight with DBH larger than 2 m. The town was established about 200 years ago and the largest *Ginkgo* tree was estimated to be 2500 years old with a DBH of 3.69 m (Li *et al*, 1999). Wuchuan County of Guizhou Province, not far from Jinfoshan, was also reported to have a natural *Ginkgo* population (Xiang and Xiang, 1997; Xiang *et al*, 1998). We sampled and analyzed the Wuchuan population. However, for unknown reasons, no amplified product was obtained with the universal primers.

Population TL, located around the border of Guizhou Province and Yunnan Province, seems to be another potential refugium because a higher than average number of haplotypes was found there and one of the haplotypes is only found there. The historical record of human colonization also supports this conclusion. It has been reported that there were 918 ancient *Ginkgo* trees in and around Tuole Village of Pan County (Xiang *et al*, 2003). The oldest is older than 1000 years, whereas the village was established about 500 years ago (Xiang *et al*, 2003).

Population SP, not far from JF and TL, is fixed for haplotype B that is not the most frequence haplotype in JF and TL. This may reflect loss of genetic variation due to a bottleneck during glaciation. Similarly, *Ginkgo* in other natural populations that may have become established after the retreat of the glaciers and bottlenecks during range expansion may explain the low haplotype diversity in these populations (Widmer and Lexer, 2001).

There are many papers discussing whether *G. biloba* population in Tianmu Mountain is natural or transplanted (Hu, 1954; Liang and Li, 2001; Lin and Zhang, 2004). In 1954, Hu, who described the living fossil *Metasequoia glyptostroboides*, reported there were some wild individuals in Zhejiang besides the planted ones (Hu, 1954). Since then, several researchers have proposed that West Tianmu Mountain was the sole refugium of *G. biloba* (Lin and Zhang, 2004), while others disagreed (Liang and Li, 2001). The present study indicates that West Tianmu Mountain does not seem to have served as a refugium for *Ginkgo*s since there is only one common cpDNA haplotype. This conclusion is in agreement with the settlement history. West Tianmu Mountain is famous for temples. The oldest Old Temple, was built in 936 AD The largest ginkgo tree is 123 cm in DBH and estimated to be about 500 years old. Hence, the Old Temple was built much earlier than the largest ginkgo tree. Of the many tree species planted around temples, the Buddhist monks preferred *Ginkgo*. As a result, there are many large ginkgo trees in or around temples in China, with some more than 1000 years old. Therefore, ginkgos in West Tianmu Mountain are likely to have been planted by monks, and may not be natural.

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